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***De novo TUBB2B* mutation causes fetal akinesia deformation sequence with  
microlissencephaly: an unusual presentation of tubulinopathy**

**Running Title:** Fetal akinesia sequence due to *TUBB2B* mutations

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**ABSTRACT**

Tubulinopathies are increasingly emerging major causes underlying complex cerebral malformations, particularly in case of microlissencephaly often associated with hypoplastic or absent corticospinal tracts. Fetal akinesia deformation sequence (FADS) refers to a clinically and genetically heterogeneous group of disorders with congenital malformations related to impaired fetal movement.

We report on an early foetal case with FADS and microlissencephaly due to *TUBB2B* mutation. Neuropathological examination disclosed virtually absent cortical lamination, foci of neuronal overmigration into the leptomeningeal spaces, corpus callosum agenesis, cerebellar and brainstem hypoplasia and extremely severe hypoplasia of the spinal cord with no anterior and posterior horns and almost no motoneurons.

At the cellular level, the p.Cys239Phe *TUBB2B* mutant leads to tubulin heterodimerization impairment, decreased ability to incorporate into the cytoskeleton, microtubule dynamics alteration, with an accelerated rate of depolymerization.

To our knowledge, this is the first case of microlissencephaly to be reported presenting with a so severe and early form of FADS, highlighting the importance of tubulin mutation screening in the context of FADS with microlissencephaly.

**KEY WORDS**

microlissencephaly, microcephaly, migration disorder, *TUBB2B*, Fetal akinesia deformation sequence

## INTRODUCTION

Normal fetal development is dependent on adequate fetal movement, starting at 8 weeks of gestation (WG). Limitation of movements results in fetal akinesia deformation sequence (FADS; OMIM 208150). FADS was first reported as a syndrome by Pena and Shokeir in 1974 and further delineated as a symptom by Hall in 1981 [1,2]. Its incidence varies among different countries and has been estimated at 1:3000 to 1:5000 by Fahy and Hall [3]. The clinical presentation is highly variable, ranging from the most severe form called lethal multiple pterygium syndrome characterized by multiple joint contractures and pterygia, lung hypoplasia, short umbilical cord, craniofacial changes consisting of hypertelorism, micrognathism, cleft palate, short neck, low-set ears, along with intrauterine growth retardation and abnormal amniotic fluid volume mainly observed from the first trimester of the pregnancy [4]. Less severe phenotypes may present either as distal arthrogryposis or as fetal hypomotility which usually occurs during the third trimester [5].

Non-genetic factors may cause FADS, such as environmental limitation of fetal movements, maternal infection, drugs and immune mechanisms (maternal autoimmune myasthenia). The FADS phenotype is observed in a number of known genetic syndromes. Non syndromic or isolated FADS is genetically heterogeneous and encompass multiple neurogenic processes affecting the central or the peripheral nervous system, the neuromuscular junction and the skeletal muscle [6-8]. Until recently, the neurogenic form characterized by spinal cord motoneuron paucity, either isolated or associated with pontocerebellar hypoplasia was considered as the most frequent cause [9-11]. Conversely, brain malformations are very infrequently observed in association with FADS, and mainly described in lissencephalies type I and II as deformations of the extremities [12,13]. To our knowledge, FADS has never been reported in association with tubulin related cortical malformations. Here, we report on the most severe presentation of tubulinopathy in a fetus harboring a *de novo* missense mutation in

the  $\beta$ -tubulin gene *TUBB2B* gene (MIM 615101), along with neuropathology and molecular data focusing on the consequences of the mutation, that could explain at least partly the severity of the lesions and early fetal presentation.

## PATIENT AND METHODS

### Case history

A 32-year-old woman, gravida 4, para 3, underwent routine ultrasonography (US) at 12 WG, which revealed severe fetal akinesia. Control ultrasound examination performed at 14 WG confirmed total lack of movements, retrognathia and dilatation of the third and fourth cerebral ventricles (supplementary figure 1). A medical termination of the pregnancy was achieved at 15 WG, in accordance with French law. A complete autopsy was performed with informed written consent from both parents. Brain lesions identified at autopsy suggested a possible Walker Warburg syndrome (WWS) despite absent eye lesions, so that a first-line screening of WWS genes was performed, but was negative. Indeed, known environmental causes of FADS were excluded, as well as syndromic causes. Chromosomal analysis performed on trophoblast biopsy revealed a normal male karyotype, 46, XY. The parents were non consanguineous and there was no relevant personal or family history. Three children born to a previous marriage were in good health.

After having obtained written informed consent from the parents, DNAs were purified from fetal lung tissues, and from peripheral blood cells in both parents by using a standard phenol/chloroform method. Mutation analysis was performed by PCR amplification and direct SANGER sequencing of all coding exons and splice sites of the *TUBB2B* gene revealed a *de novo* missense mutation in exon 4, c.716G>T determining a p.Cys239Phe substitution (previously reported in [14]). No other variant was identified after sequencing of the other genes involved in cortical malformations.

### Neuropathological evaluation

Tissues including the brain, eyes and spinal cord were fixed in a 10% formalin-zinc buffer solution. Seven-micrometer sections obtained from paraffin-embedded tissues were stained using Haematoxylin-Eosin. Adjacent brain and spinal cord sections were assessed for routine immunohistochemistry, using antibodies directed against vimentin (diluted 1:100; Dakopatts, Trappes, France), calretinin (1:200; Zymed Clinisciences, Montrouge, France), and MAP2 (diluted 1:50, Sigma, St Louis, MO). Immunohistochemical procedures included a microwave pre-treatment protocol to aid antigen retrieval (pretreatment CC1 kit, Ventana Medical Systems Inc, Tucson AZ). Incubations were performed for 32 minutes at room temperature using the Ventana Benchmark XT system. After incubation, slides were processed by the Ultraview Universal DAB detection kit (Ventana). All immunolabellings were compared with an age matched control case examined after a spontaneous abortion for premature rupture of the membranes, and whose brain was histologically normal.

## **Functional analyses**

### ***Protein modeling***

A model of human  $\beta$ -tubulin was built by homology modeling using available structures (Research Collaboratory for Structural Bioinformatics PDB code 1TUB) from Nogales et al. [15]. The images in Figure 4C were rendered using PyMOL software (<http://www.pymol.org>).

### ***Cloning and in vitro translation***

*TUBB2B* sequence was generated by PCR using a template from the human brain cDNA library (Clontech, Mountain View, CA). The PCR product was cloned into the pcDNA 3.1-V5-His vector (Invitrogen, Carlsbad, CA) and checked by DNA sequencing. These products were cloned both into the cDNA3.1-V5-his-TOPO-TA cloning vector (Invitrogen) and pET vector. An in-frame tag encoding the FLAG epitope (DYKDDDDK) was incorporated by PCR along with the C-terminus of the *TUBB2B* wild-type sequence allowing for the

distinction of the transgene from other highly homologous endogenously expressed  $\beta$ -tubulin polypeptides. The p.Cys239Phe mutation was introduced by site-directed mutagenesis using a QuikChange II kit (Stratagene, La Jolla, CA) and verified by DNA sequencing. Transcription/translation reactions were performed at 30°C for 90 min in 25  $\mu$ l of rabbit reticulocyte lysate (TNT; Promega, Madison, WI) containing  $^{35}$ S-methionine (specific activity, 1000 Ci/ $\mu$ mol; 10  $\mu$ Ci/ $\mu$ l). For the generation of labelled  $\beta$ -tubulin heterodimers, transcription/translation reactions were chased for a further 2 h at 30°C by the addition of 0.375 mg/ml of native bovine brain tubulin. Aliquots (2  $\mu$ l) were withdrawn from the reaction, diluted into 10  $\mu$ l of gel-loading buffer (gel running buffer supplemented with 10% glycerol and 0.1% bromophenol blue) and stored on ice prior to resolution on a non-denaturing gel. Labeled reaction products were detected by autoradiography after resolution on either SDS-PAGE or on native polyacrylamide gels as described [16,17].

#### ***Cell cultures, transfections and immunofluorescence***

Primary cultures of fibroblasts were derived from fibroblastic cells extracted from amniotic liquid. COS7 and Hela cells were transfected by construct with p.Cys239Phe *TUBB2B* mutation using the Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN) and grown on glass cover slips in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics. The cells were fixed with ice-cold methanol, 24–48h after growth. In the depolymerization experiments used to determine the behavioural stability of microtubules after 24 h of culture, fibroblasts were incubated for various brief intervals from 0 to 30 min on ice and fixed thereafter. Repolymerization experiments were performed by successively exposing cells at 4°C during 30mn and fixed after being incubated from 0 to 15 min at 37°C. Cells were then labelled with a polyclonal anti-FLAG antibody (1/500), or a monoclonal anti- $\alpha$ -tubulin antibody (1/1000) (Sigma-Aldrich Inc., St Louis, MO). At each experimental point, two parameters were quantified using ImageJ software: (i) the total area



of the cell and (ii) the area of the microtubules network of each cell, evaluated by quantifying the alpha-tubulin staining into the cytoskeleton and excluding the staining background corresponding to unincorporated alpha tubulin into the cellular cytoplasm. The ratio Microtubules Area/Total cell Area was used to evaluate the state of the microtubule network of the patient and control cells at each time point of depolymerisation/repolymerization experiments.

## RESULTS

### General autopsy findings

The fetus weighed 47g (50<sup>th</sup> centile). External examination disclosed cranio-facial dysmorphism with microretrognathia and cleft palate due to akinesia (supplementary figure 2), global amyotrophy and microcephaly (Figure 1A). No internal visceral malformation was found, except for the lungs which were hypoplastic, and the renal pelvis which was dilated.

### Neuropathological studies

Macroscopically, the brain appeared to be small, weighting 2.15g (5<sup>th</sup> centile, normal weight = 10g according to Guilhard-Costa and Larroche [18]). Occipito-frontal length was 21mm and transverse diameter of the cerebellum was 0.17 mm (25<sup>th</sup> centile). The brain surface was smooth, covered by thickened leptomeninges adherent to the brain (Figure 1B). Olfactory tracts were absent and the meninges seemed fused (Figure 1C). On brainstem sections, the fourth ventricle was dilated and the cerebellum seemed hypoplastic and dysplastic, resembling the cerebellar dysplasia observed in Walker Warburg syndrome (Figure 1D). Macroscopic examination of serial coronal sections confirmed the dilatation of the third and lateral ventricles, the latter being filled with congestive choroid plexuses.

Histological examination of the eyes revealed no retinal dysplasia. The spinal cord displayed major lesions consisting of severe hypoplasia and immaturity (Figure 2A). Ascending and descending tracts were missing. Anterior and posterior horns were hardly discernible, with almost no motoneurons in the anterior horns, even using MAP2 antibodies (data not shown) (Figure 2B). The cerebral mantle was particularly thin, and cortical lamination was absent, rather forming an extremely disorganized two-layered cortex extending from the inferior limit of the marginal zone to the periventricular zone with no recognizable intermediate zone (Figure 2C). Layer I was irregular in width and contained isolated or small foci of immature neurons, as well as misplaced Cajal-Retzius cells immunolabeled by calretinin antibody lying under the pial basal membrane (Figure 2D). Underneath, a single band of neurons with a vague nodular or columnar organization was found, extending to the periventricular areas (Figure 2E). The cerebral mantle was covered by fibrous meninges containing multiple dysplastic tortuous vessels with dispersed overmigrating immature neurons (Figure 2F). Vimentin immunohistochemistry showed an irregular and fragmented glia limitans (Figure 3A and B), with small gaps through which neurons overmigrated into the leptomeningeal spaces (Figure 3C, D). Vimentin immunohistochemistry also revealed severe abnormalities of the radial glia, which was virtually absent in the cortical plate and entirely disorganized in the subventricular zone (Figure 3E and F).

## Functional analyses

### *Consequences of the mutation on secondary and tertiary TUBB2B structures*

The p.Cys239 residue is located in the intermediary domain (amino acid 205–381) of the TUBB2B protein and is highly conserved during evolution among TUBB2B homologues from other species (Figure 4A). Tridimensional modeling analysis of TUBB2B using PYmol software displayed an inside localization of the variant in an helix closed to the Taxol fixation

site, apparently affecting neither the GDP binding pocket nor the  $\alpha/\beta$  interacting region (Figure 4B).

*Alteration of  $\alpha/\beta$  heterodimerization process and microtubules incorporation by TUBB2B variant*

To further investigate functional consequences of the mutation, the  $\beta$ -tubulin mutant was expressed in rabbit reticulocyte lysate and its ability to assemble into  $\alpha/\beta$  heterodimers in the presence of bovine brain tubulin was evaluated. The p.Cys239Phe mutant was translated as efficiently as the wild-type control (Figure 5A). However, analysis of the same reaction products under native conditions revealed a range of heterodimer formation that was significantly decreased both quantitatively and qualitatively in the case of p.Cys239Phe mutant compared to the wild-type control (Figure 5B) revealing an impairment of tubulin heterodimerization processes in the p.Cys239Phe mutant.

Furthermore, the transfection of the flag-tagged p.Cys239Phe *TUBB2B*-mutated construct in COS7 and HeLa cells revealed both a detectable incorporation of the protein into microtubules and in contrast to controls, a diffuse high background of label that reflects presence of unpolymerized cytosolic tubulin heterodimers, suggesting a partial impairment of the remaining heterodimer ability to incorporate into the cytoskeleton (Figure 5C).

*Effects of p.Cys239Phe TUBB2B mutation on the dynamical microtubule behavior in fetal fibroblasts*

In order to assess the behavior of the microtubules *in vivo*, we analyzed the response of the cytoskeleton to cold-induced depolymerization treatment followed by a repolymerization step at 37°C in fibroblasts extracted from amniotic liquid from control and affected patient.

Following cold-induced depolymerization, *TUBB2B* p.Cys239Phe displayed a normal rate of microtubules disintegration (Figure 5C). However, repolymerization experiments, consisting in a completed cytoskeleton depolymerization at 4°C and a gradual repolymerisation at 37°C,

revealed that p.Cys239Phe *TUBB2B* mutant microtubules are more repolymerized after 5min at 37°C than controls (Figure 5C). These experiments show that with p.Cys239Phe mutant, the repolymerization rate to renew its cytoskeleton network is accelerated, provoking a defect of the depolymerization/repolymerization balance necessary for a proper dynamic behaviour of microtubules.

## DISCUSSION

We report here the first case of Fetal Akinesia Deformation Sequence with microlissencephaly related to *TUBB2B* mutation, expanding the phenotype of ever-growing family of *tubulin* associated malformations. The diagnosis of FADS can be approached algorithmically, based on the presence of neurological symptoms and associated features [19,6]. Developmental abnormalities affecting the forebrain (e.g., hydranencephaly, microcephaly, or forebrain neuronal migration disorders), either due to primary genetic factors or to a consequence of fetal central nervous system infection or vascular insult, are sometimes associated with arthrogryposis [6,20]. In such cases, joint contractures are thought to be related to diminished corticospinal tract activation of spinal cord motor neurons. Sometimes, however, the underlying disease also directly affects spinal cord motor neurons, contributing to fetal akinesia or hypomotility. In addition to these classical causes, our report demonstrates that tubulin related microlissencephaly should be considered within the algorithm for diagnosis.

Microlissencephaly is a rare entity characterized by severe congenital microcephaly with absent sulci and gyri leading most of the time to an early fatal outcome during the foetal or the neonatal period. We have previously underlined the importance of microlissencephaly in the spectrum of tubulinopathies [14]. There are emerging molecular data to suggest that *NDE1* mutations [21,22] and more recently *KATNB1* mutations [23] are involved in the autosomal recessive forms. Tubulin mutations, particularly *TUBA1A* and less frequently

273 *TUBB2B* and *TUBB3* are significant causes of sporadic cases of microlissencephaly.

274 Tubulin related microlissencephaly share common features consisting in corpus callosum

275 agenesis, extremely reduced or absent cortical plate, hypertrophic germinal zones and

276 ganglionic eminences, hypoplastic and disorganized striatum and thalami. In our case, the

277 striatum and thalami were absent, along with germinative zones in which radial glial cells and

278 radial glia had early disappeared, representing the most severe end of the spectrum. At the

279 infratentorial level, our case also showed common signs of tubulin related

280 microlissencephaly, i.e. severe cerebellar and brainstem hypoplasia, axon tract defects with

281 absent corticospinal tracts. To our knowledge, 13 foetal cases with tubulin related

282 microlissencephaly [14,24] and 4 living patients [24,25] have been reported so far. Of these, 8

283 exhibited non specific dysmorphic features including retrognathia and hypertelorism, as well

284 as adducted thumbs, extremely long fingers, and rocker bottom feet related to poor fetal

285 mobility. The case reported here represents the extreme severe end of the spectrum due to

286 extremely severe spinal cord hypoplasia with absent anterior and posterior horns and virtually

287 indiscernible motoneurons at the histological level. In this context, fetal akinesia deformation

288 sequence undoubtedly represents a neurogenic form, reminiscent of akinesia observed in

289 Spinal Muscular Atrophy or fetal Pontocerebellar Hypoplasia type 1 [9-11].

290 The precise molecular function of *TUBB2B* in cortical development still remains unclear. Our

291 analysis suggests that the aminoacid substitution in the p.Cys239Phe *TUBB2B* mutant leads

292 to an impairment of tubulin heterodimerization processes and heterodimer ability to

293 incorporate into the cytoskeleton. Moreover, this mutant alters the microtubule dynamics with

294 an accelerated rate of repolymerization, that is has been also predicted with another *TUBB2B*

295 related microlissencephaly mutation (p.Asp249His) [26]. In the literature, we already

296 demonstrated that two *TUBA1A* and *TUBB2B* mutants' cells p.Pro263Thr and p.Ser172Pro,

297 respectively) display an opposite phenotype consisting of a defect to renew their cytoskeleton

network after total depolymerization, we assume that the two types of repolymerization impairments lead to abnormalities of the depolymerization/repolymerization balance necessary for a proper dynamic behaviour of microtubules [27,28]. As suggesting in the literature, a default in microtubules dynamics could lead to dramatic impairments of a numerous cellular processes including proliferation, migration and differentiation that are crucial steps for the brain development [28,29,30]. Therefore, we assume that this alteration of microtubule dynamics might affect brain and spinal cord development at distinct developmental steps, i.e. neurogenesis, neuronal migration and long tract formation, leading to the association of FADS and microlissencephaly. According to structural molecular models, the mutant p.Cys239Phe is predicted to alter tubulin folding. This extreme phenotype contrasts with our previous observations in which tubulin mutations predicted to impair tubulin folding but tended to be associated with milder cortical malformations [26] and emphasizes on the fact that comprehensive overview of tubulinopathies spectrum will require further investigations, including understanding of spatial and temporal consequences of tubulin mutations on MT-dependent cellular functions and early neuro-developmental processes.

In conclusion, this fetal case recapitulates the phenotypic features of tubulin related microlissencephaly expands the phenotype due to early severe arthrogryposis and underlines the importance of considering tubulin gene *TUBB2B* in the diagnosis of arthrogryposis with microlissencephaly.

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## LEGENDS TO THE FIGURES

Figure 1: Macroscopical findings

- A. Severe foetal akinesia sequence with multiple deformations, joint contractures and pterygia, along with severe global amyotrophy
- B. Superior view of the brain exhibiting a cobblestone-like appearance with abnormal meningeal vessels

C. Inferior view of the brain displaying absent interhemispheric fissure due to fusion of the meninges, absent olfactory bulbs and tracts and severely hypoplastic cerebellum

D. With absent foliation and almost indiscernible vermis and cerebellar hemispheres.

#### Figure 2: Main histological lesions

A. Severe hypoplasia of the spinal cord (arrow) (H& E stain, OMx25)

B. With at higher magnification, absent descending (arrow) and ascending tracts (triangle) and no motoneurons in the anterior horns (H& E stain, OMx250)

C. Global disorganization of the cerebral mantle with no recognizable cortical plate, intermediate and germinal zones (H& E stain, OMx25)

D. Calretinin immunohistochemistry showing aberrant Cajal Retzius cell location, with focal accumulation of these cells along the persisting glia limitans instead of forming a continuous streak located at the upper third of the molecular layer (OMx100)

E. Microscopic view of the cortical plate displaying thickened leptomeninges with tortuous vessels, only a minority of them properly penetrating into the brain parenchyma (arrow) (H& E stain, OMx100)

F. Absent lamination of the cortical plate replaced by nodules or columns of neurons, some of them overmigrating into the leptomeningeal spaces (arrow) (H& E stain, OMx250)

#### Figure 3: Vimentin immunohistochemical studies

A. Irregular thickening of the meninges (OMx100)

B. Compared with an age-matched control brain (OMx100)

C. With immature neurons accumulating just under the fragmented glia limitans (arrow) and disorganized vascular network (OMx250)

D. With abnormal fibrous walls (arrow) and absent radial glia (OMx400)

E. And paucity of radial glial cells( arrow) and fragmentation of the radial glia in the subventricular zone (OMx400)

F. Compared to an age-matched control brain where the neuroepithelium (arrow) and the subventricular zone are strongly immunolabeled (OMx400)

H&E: Haematoxylin and eosin, OM: original magnification

Figure 4: *TUBB2B* *de novo* missense mutation (p.Cys239Phe) responsible of the phenotype FADS with microlissencephaly.

A. Pedigree of the family and chromatograms showing the *de novo* occurrence of the *TUBB2B* p.Cys239Phe mutation. Square, male; round, female; diamond, fetus. Black colour, affected individual.

B. Evolution conservation of the p.Cys239 amino acid in orthologues from stickleback to human. C. Structural representation using Pymol software of the  $\alpha/\beta$  heterodimer depicted without (top left) or with (top right) surface delimitation. p.Cys239 amino acid (in green) is localized into an helix inside the monomer but outside GTP, GDP and taxol binding sites (detailed view, bottom). Pdb: 1TUB.

Figure 5: Defects in the  $\alpha/\beta$  heterodimerisation process in the microtubule incorporation and dynamical behaviour in COS7 and fibroblasts expressing the p.Cys239Phe mutation.

A. The p.Cys239Phe *TUBB2B* mutation results in inefficient  $\alpha/\beta$  heterodimer formation *in vitro*. Analysis by SDS-Page and non denaturing gel of *in vitro* translation products conducted with 35S methionine labeled wild type and p.Cys239Phe mutant. The reaction products were further chased with bovine brain tubulin to generate  $\alpha/\beta$  heterodimers. SDS-Page gel showed that the mutant was translated as efficiently as the wild type control. Note that in non denaturing gel condition, p.Cys239Phe mutant generated heterodimers in a diminished yield.

B. Expression of C-terminal Flag tagged *TUBB2B* wild type (left column) and p.Cys239Phe mutant (right column) in transfected COS7 cells revealed by anti-flag (top line) and  $\alpha$ -tubulin (bottom line) staining. Note that a part of p.Cys239Phe mutant proteins failed to incorporate into the microtubule network, producing a more diffuse cytosolic labeling pattern in comparison with WT *TUBB2B*.

C. *In vivo* analysis of cytoskeleton behaviour and stability using patient's fibroblasts from amniotic liquid. Evaluation of the sensitivity of microtubules to cold treatment and of its ability to repolymerize was analyzed in control fibroblasts and patient fibroblasts with p.Cys239Phe mutation after 8 min of cold treatment at 4°C (left graph) or after 20 min at 4°C and 5 min at 37°C (right graph). Note that the fibroblasts of the fetus correctly reacted to cold treatment by showing an increase in capacity to repolymerize compared to the wild type control.

#### **Supplementary figures**

Supplementary figure 1: US examination performed at 14 WG showing fetal (A) retrognathia and dilatation of the fourth cerebral ventricle (B)

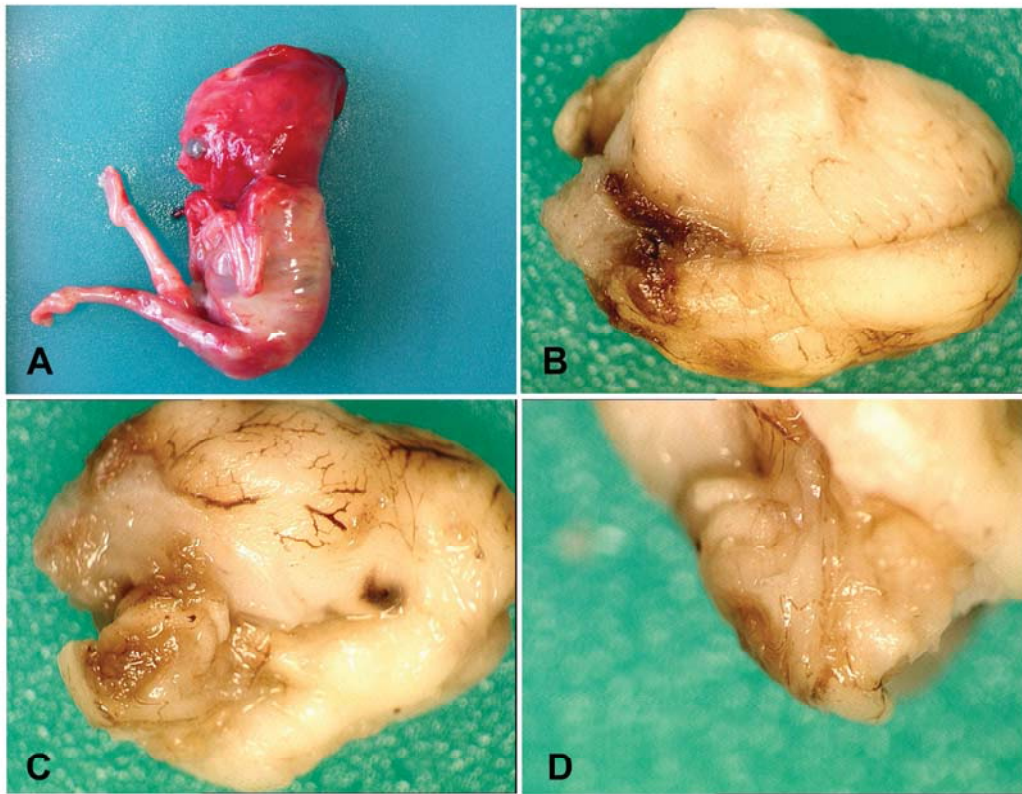
Supplementary figure 2: Fetal examination displaying arthrogryposis with clenched hands (A), posterior cleft palate (B), antebrachial C) and axillary pterygia (D).

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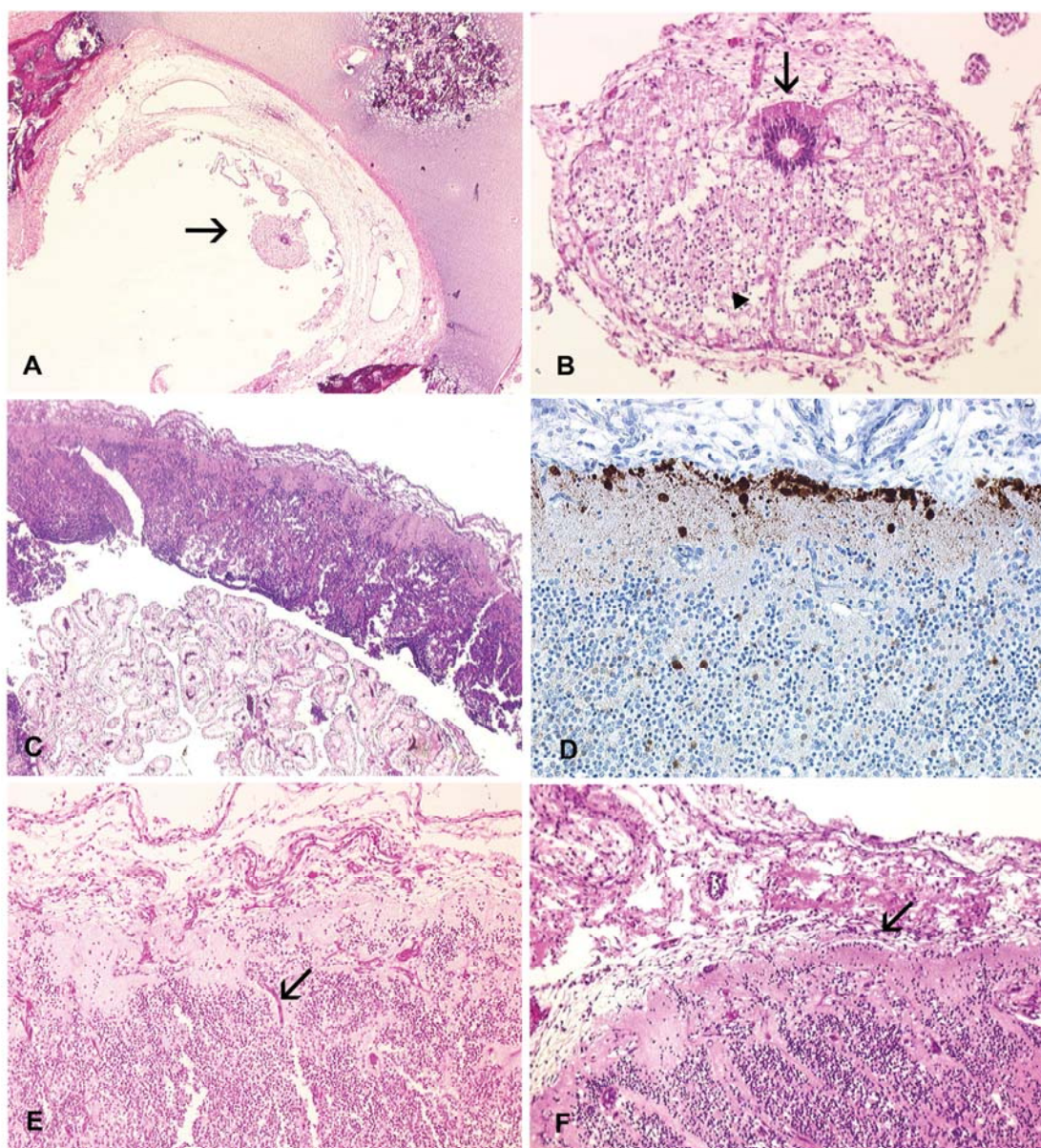
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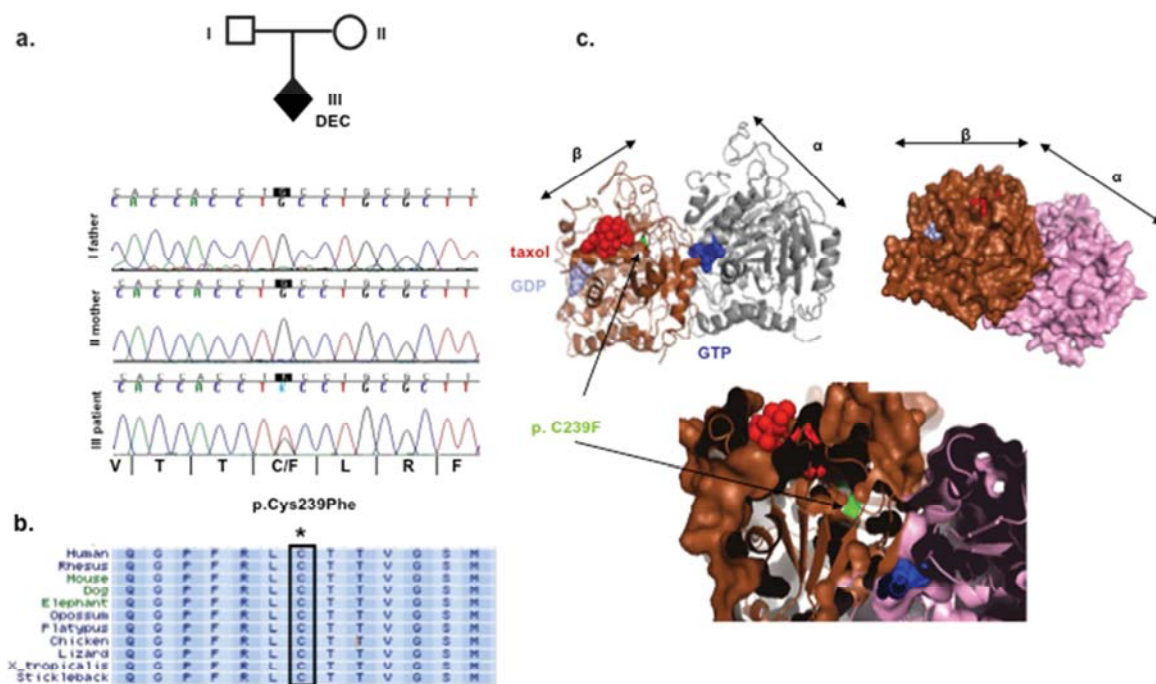


Figure 4

